Estrogenic Agonist Activity of ICI 182,780 (Faslodex) in Hippocampal Neurons: Implications for Basic Science Understanding of Estrogen Signaling and Development of Estrogen Modulators with a Dual Therapeutic Profile

Li Qin Zhao, Kathleen O’Neill, and Roberta Diaz Brinton

Department of Molecular Pharmacology and Toxicology, Norris Foundation Laboratory for Neuroscience Research, School of Pharmacy, University of Southern California, Los Angeles, California

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ABSTRACT

The present study sought to determine the characteristics of ICI 182,780 (Faslodex) action in rat primary hippocampal neurons. We first investigated the neuroprotective efficacy of ICI 182,780 against neurodegenerative insults associated with Alzheimer’s disease and related disorders. Dose-response analyses revealed that ICI 182,780, in a concentration-dependent manner, significantly promoted neuron survival following exposure to either excitotoxic glutamate (200 μM)- or β-amyloid (1.5 μM)-induced neurodegeneration of hippocampal neurons. At a clinically relevant concentration of 50 ng/ml, ICI 182,780 exerted nearly maximal neuroprotection against both insults with efficacy comparable with that induced by the endogenous estrogen 17β-estradiol. Thereafter, we investigated the impact of 50 ng/ml ICI 182,780 on mechanisms of 17β-estradiol-inducible neuronal plasticity and neuroprotection. Results of these analyses demonstrated that ICI 182,780 directly induced a series of rapid intracellular Ca2+ concentration ([Ca2+]i) oscillations in a pattern comparable with that of 17β-estradiol. In addition, ICI 182,780 exerted dual regulation of the glutamate-induced rise in [Ca2+]i, identical to that induced by 17β-estradiol. Further analyses demonstrated that ICI 182,780 induced significant activation of extracellular signal-regulated kinase 1/2 and Akt (protein kinase B) and significantly increased expression of spinophilin and Bcl-2, with efficacy comparable with neurons treated with 17β-estradiol. Taken together, results of these in vitro analyses of ICI 182,780 provide direct evidence of an estrogenic agonist profile of ICI 182,780 action in rat hippocampal neurons. Therapeutic development of neuroselective estrogen receptor modulators that mimic ICI 182,780 is discussed with respect to the potential of safe and efficacious alternatives to estrogen therapy for the prevention of postmenopausal cognitive decline and late-onset Alzheimer’s disease.

ICI 182,780 (Faslodex) is a 7α-alkylsulfinyl analog of the endogenous estrogen 17β-estradiol and binds to both estrogen receptor (ER) subtypes with a comparable affinity to 17β-estradiol (Wakeling et al., 1991). ICI 182,780 is an efficacious antagonist of the ER-dependent proliferative actions of estrogen in reproductive organs such as the breast and uterus (Howell et al., 2000). Moreover, it is a “pure” antiestrogen lacking estrogen-like agonist activity in these organs, as seen with tamoxifen (TMX) (Howell et al., 2000). Partial agonist activity of TMX is believed to be the major cause of the development of resistance to TMX therapy for the treatment of estrogen-dependent breast cancers (Howell et al., 2000). The absence of estrogenic activity and the observations that TMX-resistant tumors remain sensitive to ICI 182,780 treatment led to Food and Drug Administration approval for the use of ICI 182,780 as an adjuvant endocrine therapy to treat ER-positive metastatic breast cancers in postmenopausal women with disease progression following the first line antiestrogen therapy, i.e., TMX-resistant breast cancers (Bross et al., 2002).

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ABBREVIATIONS: ICI, ICI 182,780 (Faslodex); ER, estrogen receptor; TMX, tamoxifen; AF, activation function; SERM, selective estrogen receptor modulator; ERK, extracellular signal-regulated kinase; NBM, neurobasal medium; DIV, day(s) in vitro; HBS, HEPES-buffered saline; Akt, protein kinase B; PBS, phosphate-buffered saline; AM, acetoxymethyl ester; [Ca2+]i, intracellular Ca2+ concentration; HRP, horseradish peroxidase; NE, neuroprotective efficacy; CREB, cyclic AMP response element-binding protein; RAL, raloxifene; ICI 164,384, (N-n-butyl-N-methyl-11-[3,17β-dihydroxyestr-1,3,5(10)-trien-7α,6β]-undecanamine; E2, 17β-estradiol.
has been associated with its “down-regulation” of ER, resulting
from its disruption of the shuttling of ER between the
cytoplasm and the nucleus, a constitutively active process
involving ER in both the absence and presence of 17β-estro-
diol (Dauvois et al., 1993). The accumulated ER presence in
the cytoplasm undergoes elevated receptor turnover and deg-
radation (Linstedt et al., 1986). Inhibition of nuclear uptake
of ER induced by ICI 182,780 and its structural analogs has
been proposed to be related to the terminal portion of the 7α
side chains of these compounds, which protrude out of the
ligand binding pocket and interfere with ER dimerization, a
structural prerequisite for the translocation of ER into the
nucleus (Fawell et al., 1990). In addition, ICI 182,780 has
been demonstrated to fully abolish ER transcription regu-
lated by both activation function AF-1 and AF-2 domains,
which is distinct from the ER partial agonist/antagonist TMX
or its active metabolite, 4-hydroxyl-tamoxifen, where only
the AF-2 domain is blocked, and the estrogenic agonist ac-
tivity of these selective estrogen receptor modulators
(SERMs) can be achieved through the activation of the other
ligand-independent AF-1 domain (Wakeling et al., 1991).

Although a large number of studies demonstrated that a
high concentration of ICI 182,780 was an antiestrogen in
brain (Purves-Tyson and Keast, 2004; Miller et al., 2005),
there is a lack of research on ICI 182,780 action at a rela-
tively low pharmacological and clinically relevant concentra-
tion. Several reports indicated estrogenic activity of ICI
182,780 in select brain tissue/cells. For example, in vitro ICI
182,780 induced a significant increase in phosphorylation of
extracellular signal-regulated kinase (ERK) 1/2 in neonatal
rat primary cerebellar neurons (Wong et al., 2003). Another
in vitro study demonstrated that ICI 182,780 failed to block
the activation of ERK1/2 induced by 17β-estriadiol in basal
forebrain cholinergic neurons (Domínguez et al., 2004). The
ineffectiveness of ICI 182,780 in inhibiting 17β-estradiol-
induced agonistic effects in the brain tissue was also indi-
cated in studies on hypothalamic neurons and our own stud-
ies in hippocampal neurons (Cambiasso and Carrer, 2001).

These novel findings of “deviated” properties from the con-
nventional notion of ICI 182,780 acting as a “full” estrogen
agonist/antagonist led us to pursue in more detail the pharmacolog-
ical characteristics of ICI 182,780 action in neurons vulner-
able to neurodegeneration associated with Alzheimer’s dis-
ease and related disorders. In the present study, we sought to
determine the estrogen agonist and/or antagonist properties of
ICI 182,780 action on indicators widely accepted as key
outcomes and mechanisms of estrogenic action in hippocam-
al neurons. Results of our in vitro analyses indicate that ICI
182,780 effectively and significantly induced estrogenic out-
comes and activated underlying biochemical mechanisms
consistent with estrogen actions, supporting its estrogenic
agonist activity in these neurons.

Materials and Methods

Chemicals. 17β-Estradiol was purchased from Steraloids (New-
port, RI). ICI 182,780 was purchased from Tocris Cookson (Ellisville,
MO). The sources of other materials are indicated in the experimen-
tal methods described below.

Neuronal Cultures. The use of animals was approved by the
Institutional Animal Care and Use Committee at the University of
Southern California. Primary cultures of rat hippocampal neurons
were prepared according to the method described previously (Zhao et
al., 2004). In brief, hippocampi were dissected from the brains of
embryonic day 18 Sprague-Dawley rat fetuses, treated with 0.02%
trypsin in Hanks’ balanced salt solution (137 mM NaCl, 5.4 mM KCl,
0.4 mM KH₂PO₄, 0.34 mM NaH₂PO₄•H₂O, 10.0 mM glucose, and
10.0 mM HEPES) at 37°C for 5 min and dissociated by repeated
passage through a series of fire-polished constricted Pasteur pi-
pettes. For intracellular Ca²⁺ imaging analyses, approximately 10⁴
cells were seeded onto poly-d-lysine (10 μg/ml)-coated 22-mm cover-
slips in covered 35-mm Petri dishes. For neuroprotection and West-
en immunoblotting analyses, approximately 10⁶ cells/ml were
seeded onto poly-d-lysine-coated solid black and clear bottom 96-well
culture plates and 60-mm Petri dishes, respectively. Cells were
grown in phenol-red free neurobasal medium (NBM; Invitrogen,
Carlsbad, CA) supplemented with B27, 5 U/ml penicillin, 5 μg/ml
streptomycin, 0.5 mM glutamine, and 25 μM glutamate at 37°C in
10% CO₂ for the first 3 days and NBM without glutamate afterward.
Cultures grown in serum-free NBM yields approximately 99.5%
neu-
rons and 0.5% glial cells.

Glutamate Exposure. Primary hippocampal neurons grown on
solid black and clear bottom 96-well culture plates for 7 days in vitro
(DIV) were pretreated with vehicle alone or steroids for 48 h, fol-
lowed by exposure to 200 μM glutamate at room temperature for 5
min in HEPES-buffered saline (HBS) solution (containing 100 mM
NaCl, 2.0 mM KCl, 2.5 mM CaCl₂, 1.0 mM MgSO₄, 1.0 mM
NaH₂PO₄•H₂O, 4.2 mM NaHCO₃, 10.0 mM glucose, and 12.5 mM
HEPES). Immediately following glutamate exposure, cultures were
washed once with HBS and replaced with fresh NBM containing test
steroids. Cultures were returned to the culture incubator and al-
lowed to incubate for an additional 24 h before neuronal viability
measurements on the following day.

β-Amyloid₁–42 Exposure. β-Amyloid₁–42 (American Peptide
Company, Sunnyvale, CA) was dissolved in 10 mM HCl at 1 mM and
stored at −20°C as a stock solution. Aggregation of β-amyloid₁–42
was prepared by incubating the stock solution in 0.1 M phosphate-
buffered saline (PBS) solution (3.42 ml of 1 M NaH₂PO₄ and 1.58 ml
of 1 M NaHPO₄ in 50 ml of double-distilled H₂O) at room temper-
ature for 3 days before use, which yielded a concentration of β-amy-
loid₁–42 at 100 μM. Primary hippocampal neurons grown on solid
black and clear-bottom 96-well culture plates for 7 DIV were pre-
treated with vehicle alone or steroids for 48 h, followed by exposure
to freshly prepared 1.5 μM β-amyloid₁–42 in NBM in the presence of
vehicle alone or test steroids at 37°C for 3 days before neuronal
viability measurement.

Neuronal Viability. Neuronal viability was determined with
calcine acetoxyethyl ester (AM; Molecular Probes, Eugene, OR)
staining, which indicates the metabolically live cells in the cultures
(Zhao et al., 2004). Following glutamate or β-amyloid₁–42 exposure,
cultures were rinsed with warm PBS twice and incubated with 1 μM
calcine AM in PBS at room temperature for 30 min. For cultures
exposed to glutamate, the amount of fluorescence generated by cal-
cine, a green fluorescent product derived from enzymatic ester-
ation of calcine AM by esterases in live cells, was measured on a Spectra-
Max microplate spectrofluorometer (Molecular Devices, Sunnyvale,
CA) at excitation/emission of 485/530 nm. For cultures exposed to
β-amyloid₁–42, fluorescent micrographs were captured with a Mari-
anas digital microscopy workstation (Intelligent Imaging Innovations,
Denver, CO), and the number of live cells in the cultures
indicated by the green fluorescence staining was counted blind to the
experimental condition with the image analysis program SlideBook
4.0 (Intelligent Imaging Innovations).

Intracellular Ca²⁺ Imaging. The intracellular Ca²⁺ concentra-
tion ([Ca²⁺]ᵢ) in hippocampal neurons was measured by ratiometric
Ca²⁺ imaging with the Ca²⁺-sensitive fluorescent dye, fura-2 AM
(Molecular Probes), on an InCyto2 fluorescence imaging system (In-
tracellular Imaging, Cincinnati, OH). Before imaging, hippocampal
neurons grown on poly-d-lysine-coated coverslips for 7 DIV were
loaded with 2 μM fura-2 AM in HBS (containing 100 mM NaCl, 2.0
mM KCl, 1.0 mM CaCl₂, 1.0 mM MgCl₂, 1.0 mM NaH₂PO₄, 4.2 mM
NaHCO$_3$, 10.0 mM glucose, and 12.5 mM HEPES) at 37°C for 40 min. Excess fura-2 AM dye was removed by washing with PBS twice, and then the cultures were incubated in HBS at 37°C for 30 min to equilibrate. The coverslip with fura-2 AM-loaded neurons was mounted in a perfusion chamber placed on an inverted microscope (IMT-2; Olympus Optical, Melville, NY) equipped with epifluorescence optics (20×; Nikon, Kanagawa, Japan). The perfusion system connected to the perfusion chamber was balanced using two variable speed pumps. The cultures were perfused with HBS at a flow rate of 2 ml/min. Fura-2 AM was sequentially excited by a xenon light source at 340 and 380 nm by means of two narrow beam bandpass filters selected by a computer-controlled filter wheel. The emitted fluorescence was filtered through a 520-nm filter. A pair of images, one at 340-nm excitation and one at 380 nm, were captured with an intensified charge-coupled device camera (COHU, San Diego, CA) and analyzed with the program InCyt Im2 (Intracellular Imaging). As the [Ca$^{2+}$], rises, the fluorescence intensity generated at 340-nm excitation increases, whereas the intensity at 380-nm excitation decreases. The relative [Ca$^{2+}$], was determined by taking the ratio of emission intensity at 340- to 380-nm excitation.

Western Immunoblotting. Primary hippocampal neurons grown on poly-d-lysine-coated culture dishes for 7 DIV were treated with vehicle alone or steroids for 25 min [for analyses on ERK1/2 and protein kinase B (Akt) expression] or 48 h [for analyses on spinophilin and Bcl-2 expression]. Cultures were washed with ice-cold PBS once and scraped off the dish in 1 ml of PBS. Cells were then centrifuged at 5000 rpm for 5 min, and the pellets were dissolved in the radioimmunoprecipitation assay lysis buffer (PBS, 1% Triton, 0.2% SDS, and protease and phosphatase inhibitor cocktail set I; Calbiochem, San Diego, CA) and suspended by passage through a 200-μl pipette tip. Following incubation at 4°C for 45 min, the samples were centrifuged at 12,000 rpm for 10 min, and the supernatants were collected as the whole-cell protein extracts. Protein concentration was determined by the bicinchoninic acid method. Twenty-microgram protein samples were diluted in 15 μl of 2× SDS containing sample buffer, and the final volume was 30 μl with water. After denaturation on a hot plate at 95 to 100°C for 5 min, 20 μl of the mixture was loaded on lane on 10% SDS-polyacrylamide mini-gels followed by electrophoresis at 90 V. A high-range Precision Protein Standard (Bio-Rad Laboratories, Hercules, CA) was used to determine the protein size. The proteins were then electrotransferred to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA) from the gels. Nonspecific binding sites were blocked with 5% nonfat dry milk in PBS containing 0.05% Tween 20.

For detection of pERK1/2 expression, membranes were incubated with the primary antibody against phospho-ERK1/2 (pTpY185/187, monoclonal; Biosource International, Camarillo, CA) at 1:760 dilution in PBS-Tween at 4°C overnight and then incubated with the HRP-conjugated secondary antibody (1:3300 dilution; Vector Laboratories, Burlingame, CA) at room temperature for 1 h. After scanning, membranes were stripped and reprobed with the primary antibody against ERK2 (polyclonal, 1:5000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight followed by incubation with the HRP-conjugated secondary antibody (1:5000 dilution; Vector Laboratories) at room temperature for 1 h.

For detection of pAkt expression, membranes were incubated with the primary antibody against phospho-Akt (pS473, monoclonal; Cell Signaling Technology, Beverly, MA) at 1:2000 dilution in PBS-Tween at 4°C overnight and then incubated with the HRP-conjugated secondary antibody (1:5000 dilution; Vector Laboratories). Following, membranes were stripped and reprobed with the primary antibody against Akt (polyclonal, 1:1000 dilution; Cell Signaling Technology) at 4°C overnight followed by incubation with the HRP-conjugated secondary antibody (1:5000 dilution; Vector Laboratories) at room temperature for 1 h.

For detection of spinophilin expression, membranes were incubated with the primary antibody against spinophilin (polyclonal; Upstate, Charlotteville, VA) at 1:750 dilution in PBS-Tween at 4°C overnight and then incubated with the anti-rabbit HRP-conjugated secondary antibody (1:10,000 dilution; Vector Laboratories) at room temperature for 1 h. For detection of Bcl-2 expression, membranes were incubated with the primary antibody against Bcl-2 (monoclonal; BD Bioscience, San Jose, CA) at 1:250 dilution in PBS-Tween at 4°C overnight and then incubated with the HRP-conjugated secondary antibody (1:5000 dilution; Vector Laboratories) at room temperature for 2 h. After scanning, membranes were stripped and reprobed with the primary antibody against β-actin (monoclonal, 1:250 dilution; Santa Cruz Biotechnology) at room temperature for 2 h followed by incubation with the anti-mouse HRP-conjugated secondary antibody (1:10,000 dilution; Vector Laboratories) at room temperature for 1 h.

All the membranes were developed with a 3,3′,5,5′-tetramethylbenzidine peroxidase substrate kit (Vector Laboratories). Relative intensities of the immunoreactive bands were quantitated by optical density analysis using an image digitizing software, Un-Scan-It version 5.1 (Silk Scientific, Orem, UT).

Statistical Analyses. Data are presented as group means ± S.E.M. Statistically significant differences were determined by a one-way analysis of variance followed by a Newman-Keuls post hoc analysis.

Results

ICI 182,780 Promoted Neuronal Survival against Neurodegenerative Insults in Rat Primary Hippocampal Neurons. Multiple in vitro and in vivo studies indicate that 17β-estradiol is a highly efficacious neuroprotective agent against a wide variety of neurodegenerative insults when administered prior to the insults (Brinton, 2001). In the course of attempts to use ICI 182,780 as an estrogen antagonist in our previous experiments, we discovered that ICI 182,780 did not function as an antagonist in neurons (L. Zhao and R. D. Brinton, unpublished data). To determine in detail the estrogen agonist and/or antagonist properties of ICI 182,780 action in neurons, we first investigated the impact of ICI 182,780 on neuronal viability when challenged by neurodegenerative insults, glutamate and β-amyloid$_{1-42}$, in a prevention model paradigm using rat primary hippocampal neurons. Hippocampal neurons grown on poly-d-lysine-coated 96-well culture plates for 7 DIV were pretreated with vehicle alone or ICI 182,780 at multiple concentrations (1, 10, 50, 100, 500, and 1000 ng/ml) for 48 h before exposure to 200 μM glutamate for 5 min followed by recovery for 24 h (Fig. 1A) or 1.5 μM β-amyloid$_{1-42}$ for 3 days (Fig. 1B). 17β-Estradiol (10 ng/ml; 36.7 nM) was used as a positive control and analyzed in parallel. Neuronal viability was determined by calcein AM staining, which is indicative of metabolically active cells. Results are presented as neuroprotective efficacy (NE), which is defined as the percentage of the neurotoxin-induced cell death prevented by experimental treatment and quantitated by the equation: NE = (V$_{treatment}$ − V$_{neurotoxin}$/V$_{control}$ − V$_{neurotoxin}$) × 100%, where V$_{treatment}$ is the individual value from experimental-treated cultures, V$_{neurotoxin}$ is the mean value from neurotoxin alone-treated cultures, V$_{control}$ is the mean value from vehicle-treated control cultures, and neurotoxin refers to glutamate or β-amyloid$_{1-42}$. Exposure of neurons to either 200 μM glutamate or 1.5 μM β-amyloid$_{1-42}$ induced a significant decline in neuronal viability as demonstrated by reduced fluorescence intensity or reduced number of live cell staining in the cultures, respectively (Fig. 1, **, $P < 0.01$ compared with vehicle alone-treated control cultures). Pretreatment with ICI
both insults was comparable, and 10 ng/ml was the minimally effective concentration at which near 60% of neurons survived from both insults. Against excitotoxic glutamate, 10 ng/ml ICI 182,780 exerted the greatest neuroprotection (Fig. 1A, 51.4 ± 7.8% increase in neuronal viability compared with glutamate alone-treated cultures; **, P < 0.01), whereas 100 ng/ml was the EC_{100} against β-amyloid_{1–42}-induced cell death (Fig. 1B, 84.8 ± 12.3% increase in neuronal viability compared with β-amyloid_{1–42} alone-treated cultures; **, P < 0.01). There was no statistically significant difference in neuroprotective efficacy among 10, 50, and 100 ng/ml ICI 182,780. Based on the equal efficacy of 10, 50, and 100 ng/ml, we selected 50 ng/ml as the optimal dose for further analyses because ICI 182,780 50 ng/ml exhibited nearly maximal neuroprotection against both glutamate and β-amyloid_{1–42}-induced neurotoxicity (Fig. 1A, 39.9 ± 5.2% increase in neuronal viability compared with glutamate alone-treated cultures; **, P < 0.01; Fig. 1B, 73.6 ± 7.3% increase in neuronal viability compared with β-amyloid_{1–42} alone-treated cultures; **, P < 0.01). Moreover, 50 ng/ml ICI 182,780 induced a neuroprotective efficacy comparable with that induced by 17β-estradiol (Fig. 1A, 33.1 ± 4.2% increase in neuronal viability compared with glutamate alone-treated cultures; **, P < 0.01; Fig. 1B, 85.9 ± 8.6% increase in neuronal viability compared with β-amyloid_{1–42} alone-treated cultures; **, P < 0.01). Based on these analyses, we selected 50 ng/ml (82.4 nM), a relatively low and clinically relevant concentration of ICI 182,780 (Howell et al., 1996; Robertson et al., 2004), for subsequent comparative analyses of the impact of ICI 182,780 on the molecular mechanisms underlying estrogen-inducible neurotrophism and neuroprotection (Zhao et al., 2005).

ICI 182,780 Directly Induced a Series of Rapid But Irregular [Ca^{2+}]_i Oscillations in a Manner Comparable with 17β-Estradiol in Rat Primary Hippocampal Neurons. Although the full spectrum of molecular mechanisms that underlie estrogen promotion of neuronal plasticity and survival remains to be elucidated, a number of cellular responses have been well documented (Zhao et al., 2005). Key among these is 17β-estradiol regulation of intracellular Ca^{2+} signaling (Zhao et al., 2005). Our previous studies demonstrated that 17β-estradiol induces intracellular Ca^{2+} influx through voltage-dependent L-type Ca^{2+} channels, which is required for downstream activation of Src and mitogen-activated protein kinase signaling pathways. This signaling pathway is required for estrogen induction of neurotrophic and neuroprotective outcomes in neurons (Wu et al., 2005; Zhao et al., 2005).

In this experiment, we determined whether ICI 182,780 would directly induce a rapid rise in [Ca^{2+}]_i in neurons comparable with that induced by 17β-estradiol. Primary hippocampal neurons grown on poly-d-lysine-coated coverslips for 7 DIV were loaded with the Ca^{2+}-sensitive dye, fura-2 AM, followed by ratiometric intracellular Ca^{2+} imaging analyses. The relative change in [Ca^{2+}]_i in neurons was determined by monitoring the change in the ratio of emission fluorescence intensity at 340- to 380-nm excitation. Results of these analyses indicated that similar to with 17β-estradiol (10 ng/ml, Fig. 2A), direct perfusion of neurons with ICI 182,780 (50 ng/ml, Fig. 2B) induced a series of rapid [Ca^{2+}]_i oscillations in approximately 40 to 50% of the neurons within the 30-min observation period. Eight responsive neurons are
shown to provide a representative profile of the heterogeneity of the [Ca$_{2+}$], change in both the temporal characteristics and the magnitude of response (Fig. 2). Approximately 10 to 20% of the neurons responded at an intermediate level within the first 15 min (Fig. 2). At 15 to 30 min, both 17$eta$-estradiol and ICI 182,780-perfused cultures exhibited a greater number of responding neurons with a greater magnitude of response (Fig. 2). Overall, ICI 182,780 induced a comparable average change (0.9) in the ratio of fluorescence intensities generated at 340 and 380 nm, respectively. Throughout the 30-min observational period, approximately 50% of the neurons in the same culture responded to the steroid perfusion with unique response patterns in both response time and magnitude. For illustration, eight responding neurons from the same culture are presented to demonstrate the heterogeneity of the [Ca$_{2+}$], change among neurons. No response occurred in neurons perfused with vehicle alone-containing control HBS.

ICI 182,780 Differentially Regulated [Ca$_{2+}$], Rise in Response to Glutamate in a Manner Comparable with 17$eta$-Estradiol in Rat Primary Hippocampal Neurons. Previous findings from our group demonstrated that estrogens, including 17$eta$-estradiol and conjugated equine estrogens, exerted a dual regulation of the intracellular Ca$_{2+}$ dynamics in the presence of exogenous glutamate in neurons, depending on whether the concentration of glutamate is in the tolerable physiological range or in the excitotoxic range (Nilsen et al., 2002). At a physiological concentration of glutamate (25 M) (Nilsen et al., 2002), estrogens potentiated the glutamate-induced [Ca$_{2+}$], rise, a cellular response leading to estrogen promotion of neuronal morphogenesis and new synapse formation (Nilsen et al., 2002). In contrast, at an excitotoxic level of glutamate (200 M), estrogens attenuated the glutamate-induced [Ca$_{2+}$], rise in neurons, a cellular response considered as a fundamental event leading to estrogen promotion of neuronal defense and survival (Nilsen et al., 2002). Therefore, we pursued whether ICI 182,780 would regulate hippocampal neuron intracellular Ca$_{2+}$ dynamics in a manner comparable with estrogens. Primary hippocampal neurons grown on poly-d-lysine-coated coverslips for 7 DIV were pretreated with vehicle alone, 17$eta$-estradiol (10 ng/ml), or ICI 182,780 (50 ng/ml) for 48 h prior to ratiometric intracellular Ca$_{2+}$ imaging analyses by fura-2 AM. 17$eta$-Estradiol induced an average of 34.3% potentiation and an average 32.5% attenuation of the [Ca$_{2+}$], rise induced by 25 and 200 M glutamate, respectively (Fig. 3A, 34.3 $\pm$ 3.1% increase; Fig. 3B, 32.5 $\pm$ 3.2% reduction compared with vehicle alone-pretreated control cultures; *, $P < 0.05$; **, $P < 0.01$). ICI 182,780 induced a comparable direction and magnitude of the [Ca$_{2+}$], change with those induced by 17$eta$-estradiol. Neurons pretreated with ICI 182,780 and subsequently exposed to 25 M glutamate exhibited an average 39.1% potentiation of the [Ca$_{2+}$], rise induced by glutamate (Fig. 3A; 39.1 $\pm$ 2.8% increase compared with vehicle alone-pretreated control cultures; *, $P < 0.05$). Consistent with neuronal response to 17$eta$-estradiol, ICI 182,780 protected neurons from excess intracellular Ca$_{2+}$ with an average 32.9% attenuation of the [Ca$_{2+}$], rise induced by 200 M excitotoxic glutamate (Fig. 3B; 32.9 $\pm$ 3.9% reduction compared with vehicle alone-pretreated control cultures; **, $P < 0.01$).
ICI 182,780 Rapidly Increased ERK1/2 and Akt Phosphorylation with Efficacy Comparable with 17β-Estradiol in Rat Primary Hippocampal Neurons. One of the key downstream signaling events initiated by estrogen-induced intracellular Ca\(^{2+}\) rise and required for activation of the transcription factor, the cyclic AMP response element-binding protein (CREB), which results in increased transcription of various neurotrophic and neuroprotective genes, is the rapid activation of Src and ERK1/2 (Wu et al., 2005; Zhao et al., 2005). In parallel, through a unified upstream mechanism, estrogen binding to ER and interaction with phosphatidylinositol-3-kinase activates the Akt signaling pathway, which plays a pivotal role in altering the expression and function of the Bcl-2 family proteins (Znamensky et al., 2005). We determined whether ICI 182,780 would activate these same signaling mechanisms in rat primary hippocampal neurons. Neurons grown on poly-D-lysine-coated culture dishes for 7 DIV were B27 supplement-deprived for 45 min prior to incubation with vehicle alone, 17β-estradiol (10 ng/ml), ICI 182,780 (50 ng/ml), or 17β-estradiol (10 ng/ml) plus ICI 182,780 (50 ng/ml) for 25 min prior to harvesting of proteins for detection of phosphorylated ERK and Akt expression by Western immunoblotting analyses. Total ERK and Akt expression levels in the same protein samples were also detected and used as the loading controls. Results of these analyses indicate that exposure of neurons to ICI 182,780 rapidly induced a significant increase in phosphorylation of both ERK2 and Akt (Fig. 4A, 28.6 ± 5.6%; Fig. 4B, 33.1 ± 5.0% increase compared with vehicle alone-treated control cultures, respectively; **, \(P < 0.01\)), with efficacy comparable with that induced by 17β-estradiol (Fig. 4A, 55.0 ± 13.6%; Fig. 4B, 25.8 ± 2.3% increase compared with vehicle alone-treated control cultures, respectively; **, \(P < 0.01\)). The presence of ICI 182,780 failed to block the effect of 17β-estradiol and combined use of both steroids exerted a comparable impact on both pERK1/2 and pAkt (Fig. 4A, 43.0 ± 9.6%; Fig. 4B, 30.9 ± 6.9% increase compared with vehicle alone-treated control cultures, respectively; **, \(P < 0.01\)). There were no statistically significant differences among the 17β-estradiol, ICI 182,780, and 17β-estradiol plus ICI 182,780 treatment groups.

ICI 182,780 Increased Spinophilin and Bcl-2 Expression with Efficacy Comparable with 17β-Estradiol in Rat Primary Hippocampal Neurons. Spinophilin, a protein that is enriched in the heads of neuronal dendritic spines, has been demonstrated to play a significant role in modulating both dendritic morphology and glutamatergic synaptic activity (Feng et al., 2000; Lee et al., 2004). We have shown previously that activation of CREB initiated by estrogen activation of the intracellular Ca\(^{2+}\)/Src/ERK signaling cascade led to increased expression of spinophilin in hippocampal neurons, which is consistent with estrogen-inducible promotion of neuronal morphogenesis and synaptogenesis (Zhao et al., 2005).

In light of these findings, we conducted Western immunoblotting analyses to determine the impact of ICI 182,780 on the expression of spinophilin in rat primary hippocampal neurons. We used β-actin as an internal loading control in our analyses because we determined that β-actin does not change in response to either 17β-estradiol or progesterone even though β-actin is enriched in spines and interacts with spinophilin. Hippocampal neurons grown for 7 DIV and treated with 50 ng/ml ICI 182,780 for 48 h had a significant increase in spinophilin expression (Fig. 5A, 44.8 ± 8.9% increase compared with vehicle alone-treated control cultures; **, \(P < 0.01\)), which was statistically comparable with that induced by 10 ng/ml 17β-estradiol (Fig. 5A, 29.5 ± 7.4%
increase compared with vehicle alone-treated control cultures; *, $P < 0.05$). Coadministration of 17β-estradiol (10 ng/ml) and ICI 182,780 (50 ng/ml) resulted in a comparable effect with that induced by either 17β-estradiol or ICI 182,780 alone (Fig. 5A, 36.0 ± 8.1% increase compared with vehicle alone-treated control cultures; **, $P < 0.05$).

Estrogen activation of both intracellular Ca$^{2+}$/Src/ERK/CREB and Akt signaling cascades leads to up-regulation of the Bcl-2 family antiapoptotic proteins, such as Bcl-2 and Bcl-xl, has been proposed as a critical component underlying estrogen promotion of neuronal survival (Nilsen and Brinton, 2003). Estrogen-inducible elevation in Bcl-2 and Bcl-xl expression in neurons enhances mitochondrial Ca$^{2+}$ load tolerability induced by neurotoxic insults, leading to estrogen neuroprotection through sustained mitochondrial function (Nilsen and Brinton, 2003).

Based on these earlier findings, we evaluated the impact of ICI 182,780 on expression of Bcl-2 protein in rat primary hippocampal neurons. Neurons grown for 7 DIV were treated with vehicle alone, 17β-estradiol (10 ng/ml), ICI 182,780 (50 ng/ml), or 17β-estradiol (10 ng/ml) plus ICI 182,780 (50 ng/ml) for 48 h followed by Western immunoblotting analyses for spinophilin and Bcl-2 expression in whole-cell lysate preparations. β-Actin was used as an internal loading control protein. Results are presented as percent increase in the test protein expression compared with vehicle alone-treated control cultures and expressed as mean ± S.E.M., $n \geq 3$; **, $P < 0.01$.
ICI 182,780 was protective against diol-induced neural responses. Moreover, present as an additional structure absent in both TMX and raloxifene (RAL), ICI 182,780 should also be classified as a mixed estrogen agonist/antagonist. Based on this observation, it is conceivable that there might exist ER variants (e.g., with the structural alteration in AF-2) in neurons; binding of ICI compounds could induce the repositioning of helix 12 distinct from that observed in the full-length receptor and lead to exposure of the AF-2 without affecting the dimerization and nuclear uptake capability of the receptors.

Alternatively, studies from multiple laboratories, including our own, indicate that a membrane-associated estrogen-binding receptor induces the rapid actions of 17β-estradiol and ICI 182,780 in neurons (Brinton, 2001; McEwen, 2002). Based on our recent observations that both ERα-selective agonist PPT and ERβ-selective agonist DPN were effective in activating the neurotrophic and neuroprotective outcomes and the same underlying mechanisms, we propose that this membrane-associated estrogen-binding receptor could be ERα or ERβ or their structural analogs (Zhao et al., 2004). This hypothesis is partially supported by increasing evidence indicating the subcellular localization of ERα and ERβ in non-nuclear compartments including membranes in rat and mouse cortical and hippocampal neurons (Milner et al., 2001; Nishio et al., 2004; Kalita et al., 2005). In addition, our observation that PPT and DPN compete for the same membrane binding site as the BSA-17β-estradiol conjugate (BSA-17β-estradiol-fluorescein isothiocyanate) in rat hippocampal neurons provides indirect evidence for membrane localization of ERα and ERβ or at least ERα and ERβ-related receptors (Wu et al., 2005). Functionally, it is likely that these membrane-associated classical ER-related receptors could associate and act with other membrane structures, leading to initiation of signaling cascades (Norman et al., 2004; Mannella and Brinton, 2006).

An alternative hypothesis for a membrane-associated estrogen-binding receptor in neurons could be a structurally distinct class of receptors independent of the classical ER. In support of this hypothesis, recent discoveries demonstrated that a transmembrane G protein-coupled receptor, GPR30, although it remains controversial whether it localizes to the plasma membrane or exclusively to the endoplasmic reticulum, can be activated by a number of the same ER ligands.
and responsible for estrogen-induced rapid signaling independent of classical ER in some cell types, including ER-negative cell types (Revankar et al., 2005; Thomas et al., 2005). ICI 182,780 exhibits high binding affinity to GPR30 and acts as an agonist of GPR30 (Thomas et al., 2005), which has important implications for a membrane-associated estrogen-binding receptor in mediating the consistent agonistic effects of 17β-estradiol and ICI 182,780 in hippocampal neurons.

From a therapeutic development perspective, our findings that ICI 182,780 acts as an estrogen agonist in neurons provides a translational opportunity for development of an ideal brain-selective ER modulator that mimics the beneficial effects of estrogen agonists in brain while lacking or antagonizing activation of estrogenic proliferative responses in reproductive organs (neuro-SERMs) (Zhao et al., 2005). Because ICI 182,780 does not readily cross the blood-brain barrier (Howell et al., 2000), design and development of a brain-accessible ICI-like molecule that is structurally and functionally analogous to ICI 182,780 while possessing improved chemical features that promote blood-brain barrier penetration is required. The availability of such a molecule will provide a valuable pharmacological tool to further decipher the in vivo activity of this category of ER ligands in the brain. Proof of principle of the in vivo efficacy of ICI to mimic neuro-SERMs is anticipated to provide a dual therapeutic profile to treat ER-positive breast cancers while simultaneously preventing estrogen deficiency-associated cognitive decline and neurodegeneration in postmenopausal women.

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References


Address correspondence to: Dr. Roberta Diaz Brinton, Molecular Pharmacology and Toxicology, Neuroscience, and Biomedical Engineering, University of Southern California, 1985 Zonal Avenue, PSC 503, Los Angeles, CA 90089. Email: rbrinton@usc.edu